Interactions between transgenic trees and mycorrhizal and pathogenic fungi

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ABBREVIATIONS USED

BAP	6-benzylaminopurine
Bp	*Betula pendula*
***Bt*(*k*)**	*Bacillus thuringiensis* (var. *kurstaki*)
CAD	cinnamyl alcohol dehydrogenase
CAld5H	coniferaldehyde 5-hydroxylase
CaMV	cauliflower mosaic virus
CCoAOMT	caffeoyl coenzyme A O-methyltransferase
CCR	cinnamoyl-CoA reductase
cDNA	complementary DNA
C3H	coumarate 3-hydroxylase
C4H	cinnamate 4-hydroxylase
4CL	4-coumarate:coenzyme A ligase
CoA	coenzyme A
COMT	caffeic acid *O*-methyltransferase
CTAB	cetyl trimethyl ammonium bromide
dCTP	deoxycytidine triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
ECM	ectomycorrhiza
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
F5H	ferulate 5-hydroxylase
G unit	guaiacyl unit of lignin
GM	genetically modified
H unit	p-hydroxyphenyl unit of lignin
HGT	horizontal gene transfer
IAA	indole-3-acetic acid
MOPS	4-morpholino propanesulfonic acid
MS	Murashige & Skoog Medium
MSG	modified Murashige & Skoog Medium
MWL	milled wood lignin
npt	neomycin phosphotransferase

PAL	Phe ammonia-lyase
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
Q-HSQC	quantitative heteronuclear single quantum coherence
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
S unit	syringyl unit of lignin
SDS	sodium dodecyl sulphate
SIR	substrate induced respiration
SSC	sodium chloride / sodium citrate
SSTE	sodium chloride / SDS / Tris-HCl / EDTA
STS	stilbene synthase
TE	Tris-HCl /EDTA
WPM	woody plant medium

ABSTRACT

The development of biotechnology techniques in plant breeding and the new commercial applications have raised public and scientific concerns about the safety of genetically modified (GM) crops and trees. To find out the feasibility of these new technologies in the breeding of commercially important Finnish hardwood species and to estimate the ecological risks of the produced transgenic plants, the experiments of this study have been conducted as a part of a larger project focusing on the risk assessment of GM-trees. Transgenic *Betula pendula* and *Populus* trees were produced via *Agrobacterium* –mediated transformation. Stilbene synthase (STS) gene from pine (*Pinus sylvestris*) and chitinase gene from sugar beet (*Beta vulgaris*) were transferred to (hybrid) aspen and birch, respectively, to improve disease resistance against fungal pathogens. To modify lignin biosynthesis, a 4-coumarate:coenzyme A ligase (4CL) gene fragment in antisense orientation was introduced into two birch clones. In *in vitro* test, one transgenic aspen line expressing pine STS gene showed increased resistance to decay fungus *Phellinus tremulae*. In the field, chitinase transgenic birch lines were more susceptible to leaf spot (*Pyrenopeziza betulicola*) than the non-transgenic control clone while the resistance against birch rust (*Melampsoridium betulinum*) was improved. No changes in the content or composition of lignin were detected in the 4CL antisense birch lines.

In order to evaluate the ecological effects of the produced GM trees on non-target organisms, an *in vitro* mycorrhiza experiment with *Paxillus involutus* and a decomposition experiment in the field were performed. The expression of a transgenic chitinase did not disturb the establishment of mycorrhizal symbiosis between birch and *P. involutus in vitro*. 4CL antisense transformed birch lines showed retarded root growth but were able to form normal ectomycorrhizal associations with the mycorrhizal fungus *in vitro*. 4CL lines also showed normal litter decomposition. Unexpected growth reductions resulting from the gene transformation were observed in chitinase transgenic and 4CL antisense birch lines.

These results indicate that genetic engineering can provide a tool in increasing disease resistance in Finnish tree species. More extensive data with several ectomycorrhizal species is needed to evaluate the consequences of transgene expression on beneficial plant-fungus symbioses. The potential pleiotropic effects of the transgene should also be taken into account when considering the safety of transgenic trees.

1. INTRODUCTION

1.1. GENETIC ENGINEERING OF FOREST TREES

In conventional tree breeding, genetic improvement of forest trees is based on the natural genetic variation of economically important traits. Forest tree breeding has focused on quantitative traits controlled by several genes and it has been hampered for the mating system (self-incompatibility and high degree of heterozygosity) and the biology of forest trees, namely long age and slow maturation. As a result, many tree improvement efforts have been directed at identifying superior individuals and propagating them clonally. Genetic engineering of forest trees however, allows the modification of single traits in selected genotypes without affecting the genetic background of the tree. As the biotechnological methods for *in vitro* propagation and genetic transformation have progressed, forest trees have become important targets for genetic engineering (Peña and Séguin 2001, Campbell et al. 2003). Due to their economic importance, biotechnology research of trees has focused on coniferous forest trees, yet the most progress with the transformation has been accomplished with hardwood species. Early reports on the genetic transformation of forest trees have been concentrated on the genus *Populus* and even today, *Populus* remains the principal genetically transformed tree species (Merkle and Nairn 2005). Compared to crop plants, the domestication of forest trees is still in its infancy, but the application of biotechnology offers a great potential to accelerate tree improvement programs (Campbell et al. 2003).

The targets for forest tree engineering mostly aim to improve the volume or quality of wood produced. By introducing sterility into genetically modified trees, resources could be directed for vegetative growth and the gene flow through pollen and seed could be prevented. In trees, the prevention of flowering has been shown in early-flowering birch clones expressing the ribonuclease gene BARNASE ligated to the flower specific promoter (Lemmetyinen et al. 2004). To shorten the long juvenile phase of trees, early flowering has been achieved in transgenic trees constitutively expressing flower-meristem-identity genes. Shorter flowering times lead to shorter generation times which in turn allow acceleration of breeding programmes (Weigel and Nilsson 1995, Peña et al. 2001). Manipulation of plant hormone levels in order to promote growth resulted in

transgenic *Populus* trees with faster growth, longer xylem fibers and increased biomass (Eriksson et al. 2000). Insects are responsible for substantial losses in forest tree species and their damage can sometimes be a limiting factor for tree growth and survival. Different approaches have been employed in the production of trees resistant to herbivores. The bacterium *Bacillus thuringiensis* (*Bt*) toxins have been used in crop species as well as in forestry for genetic engineering of insect-resistant plants (Schuler et al. 1998). In addition, significant levels of resistance to insect damage have been achieved in transgenic *Populus* expressing proteinase inhibitors (Leplé et al. 1995, Delledonne et al. 2001). Transgenic trees have also been generated for herbicide resistance to reduce the economical cost of weed control (Campbell et al. 2003) and for remediation of contaminated soils (Rugh et al. 1998). However, most efforts in genetic engineering of forest trees have been directed to improving wood properties and disease resistance, which will be discussed in the following paragraphs.

1.2. MANIPULATION OF WOOD PROPERTIES

Because of the increasing global demands for pulp, paper and timber products, many tree biotechnology studies have focused on altering the quality or quantity of wood. The wood is primarily composed of cellulose, hemicelluloses, and lignin. Lignin is a complex phenolic polymer found in the cell walls of some plant cells such as secondary walls of xylem vessels. It provides compressive strength and renders the walls hydrophobic and impermeable enabling the transport of water and solutes through the vascular system. Lignin is needed for mechanical support in terrestrial plants and it also plays a role in protecting plants against pathogens (Whetten and Sederoff 1995, Campbell and Sederoff 1996, Whetten et al. 1998, Boerjan et al. 2003). Despite of the importance of lignin to plant growth, in the conversion of wood into pulp and paper, lignin is an undesirable wood component. The extraction of lignin from cellulose is costly and requires large quantities of chemicals therefore, it would be highly desirable to manipulate wood to contain less lignins or to make lignins more extractable.

The pathway of lignin biosynthesis is complex and even after several decades of intensive research work some aspects of its biosynthesis still remain unclear (Figure 1). The structure of lignins varies between plant species, cell types within a plant, and between different parts of a single cell wall. Lignins are considered to be polymers of three

hydroxycinnamyl alcohol monomers (monolignols): p-coumaryl, coniferyl, and sinapyl alcohol which produce, respectively, p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units when incorporated into the lignin polymer. Monolignols are derived from phenylalanine in a multistep process and differ in the extent of methoxylation. Although exceptions exist, dicotyledonous angiosperm (hardwood) lignins consist principally of G and S units and traces of H units, whereas gymnosperm (softwood) lignins are composed mostly of G units with low levels of H units (Whetten and Sederoff 1995, Whetten et al. 1998, Boerjan et al. 2003). In the lignin molecule, monomeric units are linked together by different bond types. The most frequent linkage is the β-O-4 linkage which is labile and most easily cleaved chemically. The other linkages are β-5, β-β, β-1, 5-5, and 4-O-5 linkages, which are more resistant to chemical degradation (Boerjan et al. 2003, Jouanin and Goujon 2004). Lignins composed mainly of G units, such as conifer lignins, contain more resistant linkages than lignins incorporating S units (Boerjan et al. 2003), which makes the lowering of the amount of G units a desirable target for genetic engineering. The biochemical pathways for syringyl monolignol biosynthesis have for long remained ambiguous and only recently have the enzymes involved in its biosynthesis been demonstrated in aspen (Li et al. 2001).

Genetic modification of the lignin biosynthetic pathway has been primarily studied in transgenic plants obtained via sense or antisense strategies. Most of the genes involved in lignin biosynthesis have been manipulated in model species *Arabidopsis* and tobacco, and some experiments have been performed in trees such as poplar. In trees, there are examples of successful attempts to modify the subunit composition of lignin in favour of the more extractable S units. *Populus tremuloides* trees expressing sense coniferaldehyde 5-hydroxylase (CAld5H) showed S/G ratio increases as much as 3-fold without lignin quantity change (Li et al. 2003). Transgenic poplar trees carrying the ferulate 5-hydroxylase (F5H) under a tissue specific promoter also displayed enhanced lignin syringyl content (Franke et al. 2000). Antisense or sense transformation of genes encoding lignin biosynthetic enzymes has also been effective at reducing lignin quantity. Down-regulation of COMT (Jouanin et al. 2000), caffeoyl coenzyme A O-methyltransferase (CCoAOMT) (Zhong et al. 2000) and peroxidase activity (Morohoshi and Kajita 2001) caused a decrease in lignin content in transgenic poplars. Improved pulping properties due to the slight decrease in lignin content and changes in lignin structure (higher content of free phenolic units) were detected in field trials of cinnamyl

alcohol dehydrogenase (CAD) suppressed poplars (Pilate et al. 2002). Manipulating the expression of lignin biosynthetic genes and the analysis of transgenic plants has contributed to our understanding of the structure and biosynthesis of lignin.

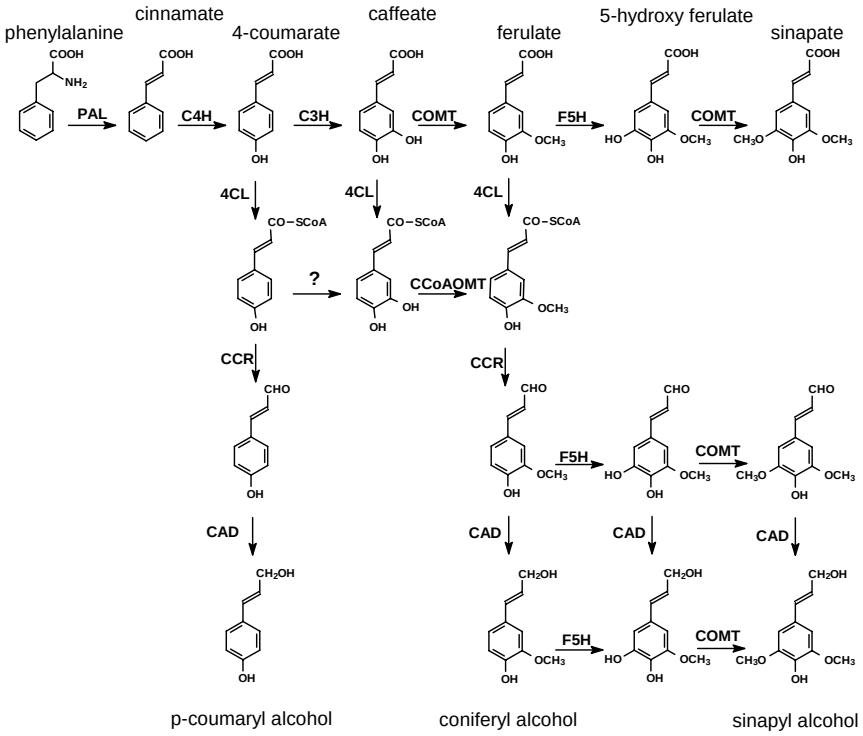

Figure 1. An outline of the monolignol synthetic pathway. CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; C3H, coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; COMT, caffeic acid *O*-methyltransferase; F5H, ferulate 5-hydroxylase; PAL, Phe ammonia-lyase. Figure by courtesy of Teemu H. Teeri.

1.2.1. 4-coumarate:coenzyme A ligase (4CL)

Some of the most drastic changes in lignin quantity have been seen in transgenic trees with modified expression of the 4CL (4-coumarate:coenzyme A ligase). 4CL is an

enzyme that converts 4-coumaric acid and other substituted cinnamic acids such as caffeic and ferulic acid into the corresponding CoA thiol esters, which are used for the biosynthesis of different phenylpropanoid-derived compounds including flavonoids, coumarins, stilbenes, and lignin (Gross and Zenk 1966, Gross and Zenk 1974, Hahlbrock and Scheel 1989). In general, 4CL has high or intermediate substrate specificity for 4-coumaric acid, ferulic acid and caffeic acid but low or not detectable affinity for cinnamic acid or sinapic acid (Lofty et al. 1989, Voo et al. 1995, Allina et al. 1998, Ehlting et al. 1999). 4CL genes generally exist in plants as a multigene family and it has been proposed that isoforms might control the formation of different phenylpropanoid products (Grand et al. 1983, Uhlmann and Ebel 1993, Hu et al. 1998, Ehlting et al. 1999, Lindermayr et al. 2002). In angiosperms, phylogenetic comparisons divide 4CL proteins into two major clusters, Class I and Class II (Ehlting et al. 1999). Class I proteins are closely related to the biosynthesis of lignin and other phenylpropanoids while Class II 4CLs have been associated with flavonoid biosynthesis (Cukovic et al. 2001). Transgenic *Populus tremuloides* trees with an antisense expression of a gene encoding *Pt*4CL1 exhibited up to 45% reduction of lignin, 15% increase in cellulose, and growth enhancement of transgenic plants (Hu et al. 1999). The most dramatic repression of lignin biosynthesis has been achieved in aspen cotransformed with antisense 4CL and sense CAld5H having up to 52% less lignin, 30% more cellulose, and a modified subunit composition in favour of S units (Li et al. 2003). Genetic manipulation of 4CL thus could be a promising strategy for reducing lignin content to improve wood-pulp production efficiency.

1.3. DISEASE RESISTANCE

Plants are continuously exposed to pathogens like viruses, fungi or bacteria and have evolved a number of strategies to resist infection. These defence mechanisms include preformed physical barriers such as cell wall or cuticle and the active defence mechanisms induced by a potential pathogen (Preisig-Müller et al. 1999). Typical defence reactions exhibited by plants towards microbial infection are either localized or systemic and include the collapse of challenged plant cells (hypersensitive response), the production of reactive oxygen species, the activation of defence related genes, structural changes in the cell walls (e.g. increased lignification) and the synthesis of phytoalexins (Ebel and Mithöfer 1998). Various novel proteins referred to as pathogenesis-related proteins (PRs) are induced as part of the plant's defensive responses. Chitinases, β-1,3-

glucanases, proteinase inhibitors, and peroxidases are examples of PRs with potential antimicrobial activity (van Loon and van Strien 1999).

The traditional breeding for resistance in forest trees is based on either single gene effects or multiple resistance alleles; the resistance can also be caused more by general tree health than any specific mechanism. In tree breeding, a need for stable resistance is obvious as genotypes remain in the environment for a long period of time compared to the short generation cycles of many pathogens. Regardless of whether conventional breeding or single gene transgenic introduction techniques are used, it is not clear that genes can be selected that would create an enduring resistance (Namkoong 1991). Breeding programmes of many crop species have shown that the resistance being overcome by genetic shifts in pathogen populations is usually associated with resistance conferred by single major genes (R genes) and the most durable resistance against bacterial or fungal pathogens have involved complex, multiple resistance factors. In the prevention of the evolution of pathogen populations adapted to the new resistance mechanisms, it is not the GM versus non GM-status of the plants that may result in disease resistance breakdown, but the way the plants are grown and managed (Namkoong 1991, Conner et al. 2003).

Different biotechnological strategies for improving the resistance of trees to viruses, bacteria, and fungi have been experimented. Resistance against virus disease was shown in transgenic plum expressing the coat protein gene of plum pox virus through the mechanism of post-transcriptional gene silencing (Scorza et al. 2001). A significant reduction of symptoms caused by the necrotic bacterium *Erwinia amylovora* was observed in transgenic pear containing a lytic protein attacin of insect origin (Reynoird et al. 1999). Fungi can be considered as the most important pathogens of trees. Two of the most famous examples of trees devastated by fungal diseases are the chestnuts (*Castanea*) and elms (*Ulmus*). The chestnut blight fungus (*Cryphonectria parasitica*) and the fungus *Ophiostoma ulmi* causing Dutch elm disease have incurred substantial ecological and commercial losses both in Europe and North America. Elm and chestnut plantlets transformed with antifungal genes have been produced and their ability to resist these diseases is being tested (Merkle and Nairn 2005). Examples of the successful introduction of fungal resistance into tree species using genetic engineering include hybrid poplars expressing a wheat oxalate oxidase gene showing enhanced resistance to

poplar pathogenic fungus *Septoria musiva* (Liang et al. 2001) and tree species transformed with chitinase genes (see paragraph 1.3.1). Only time and commercial applications will verify if these genetically modified fruit and forest trees demonstrate strong and stable field resistance against evolving pathogen populations.

1.3.1. Chitinase

Chitinases are examples of antifungal cell wall degrading enzymes that have been successfully used in genetic engineering of various economically important plant species in order to improve plants' resistance against fungal diseases (Broglie et al. 1991, Grison et al. 1996, Lorito et al. 1998, Tabei et al. 1998). Induction of chitinases is considered to have an important role as a part of the general defence response in plants since apart from fungal pathogens, induction can occur by bacteria, viruses and various physical, chemical, and environmental stresses (Sahai and Manocha 1993). Chitinases are able to catalyse the hydrolysis of chitin, a homopolymer of L-1,4-N-acetyl-D-glucosamine and a primary structural component of the cell wall of all true fungi and arthropod exoskeleton (Bartnicki-Garcia 1968). They may act directly by causing swelling and lysis of hyphal tips and blocking the growth of the invading hyphae (Schlumbaum et al. 1986, Mauch et al. 1988, Vierheilig et al. 2001) or indirectly as well by releasing fungal elicitors which then induce additional chitinase activity and other defence reactions in the host (Roby et al. 1987, Barber et al. 1989). Besides acting in plant defence reactions, chitinases may have a role in functions related to plant growth such as cell division, differentiation, and development (Collinge et al. 1993, Sahai and Manocha 1993, Patil and Widholm 1997).

Not only plants contain chitinases; bacteria, fungi and insects are also known to synthesize chitinases for biopesticidal purposes (Kramer and Muthukrishnan 1997). Chitinases of fungal origin have been applied to engineering disease resistance in trees. Endochitinase from the biocontrol fungus *Trichoderma harzianum* increased the resistance of transgenic apple to apple scab (Bolar et al. 2000). Recently, transgenic lines of black spruce and hybrid poplar expressing *T. harzianum* endochitinase gene were shown to demonstrate an increased resistance to the spruce root pathogen *Cylindrocladium floridanum* and to the leaf rust pathogen *Melampsora medusae*, respectively (Noël et al. 2005). Plant chitinases are classified into six classes based on amino acid sequence features (Neuhaus et al. 1996, Brunner et al. 1998), and there are indications that various isoforms of

chitinases are differentially regulated at the level of gene expression (Collinge et al. 1993, Salzer et al. 2000). The most extensively studied plant chitinases are endochitinases which randomly hydrolyse internal β-1,4-linkages releasing chitin oligosaccharides; exochitinases catalyse the release of N-acetylglucosamine residues from chitin polymer (Boller et al. 1983, Collinge et al. 1993). A sugar beet (*Beta vulgaris*) chitinase gene belonging to class IV chitinases has been introduced into a commercially important, deciduous tree, silver birch (*Betula pendula* Roth) (Pappinen et al. 2002). Basic class IV endochitinase from sugar beet is synthesized without a C-terminal vacuolar targeting sequence and is deposited extracellularly in the apoplast (Mikkelsen et al. 1992) and its antifungal activity against a fungal pathogen *Heterobasidion annosum* has been demonstrated *in vitro* (Susi et al. 1995). In a greenhouse experiment, the transgenic birch lines showing high levels of sugar beet chitinase IV expression were more resistant to the fungal pathogen *Pyrenopeziza betulicola* (Fuckel) than the non-transgenic control clone (Pappinen et al. 2002).

1.3.2. Stilbene synthase (STS)

Stilbenes, as well as the lignins, are products of the phenylpropanoid pathway often related to natural resistance in plants. They are phenolic compounds, which contain two benzene rings separated by an ethene (Gorham 1995), known to be toxic to bacteria and mice (Frykholm 1945), insects (Wolcott 1951), fish (Erdtman 1939) as well as many fungi (Rennerfelt 1943, 1945, Rennerfelt and Nacht 1955, Hart 1981). Stilbenes have been isolated from a wide range of unrelated plant families including *Pinus*, *Picea*, *Eucalyptus*, and *Vitis* species (Gorham 1995). They are produced in plants as stress metabolites and also as constitutive defensive agents in lignified tissues (Hart 1981). The most wide-spread stilbenes are resveratrol and its derivatives which are common in e.g. *Vitis* while pinosylvin and its derivatives are confined to *Pinus* species (Schröder et al. 1993). The prospects for using a gene encoding for a stilbene synthase (STS) in genetic engineering of disease resistance are promising since all higher plants are able to synthesise its precursors, and the ability to synthesise stilbenes is thought to be dependent solely on the presence of STS (Hain et al. 1990). Stilbene synthases can be grouped into two categories. The enzymes from grapevine, peanut and *Eucalyptus* prefer 4-coumaroyl-CoA as a substrate and form resveratrol, while pinosylvin synthase, the STS in pine, uses cinnamoyl-CoA to synthesise pinosylvin (Schwekendiek et al. 1992) (Figure 2).

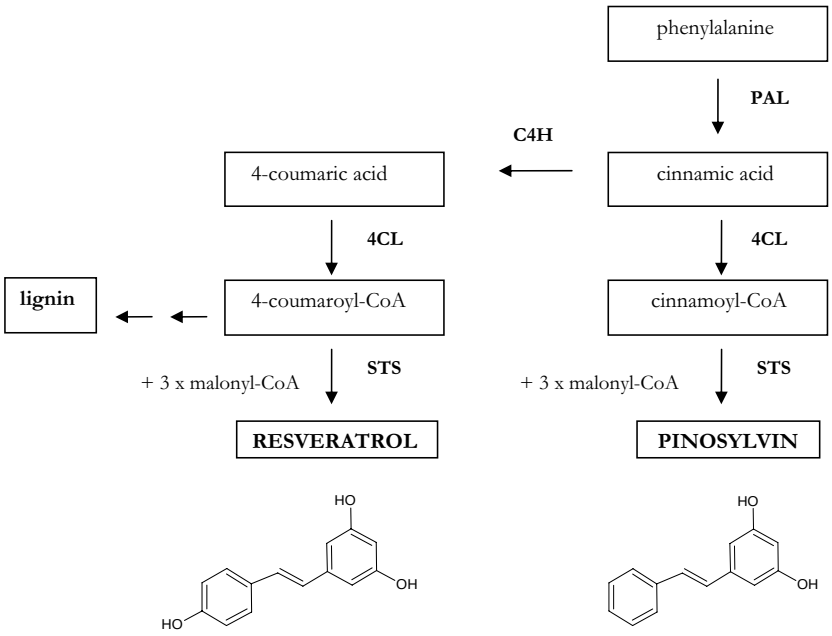

Figure 2. Biosynthesis of stilbenes resveratrol and pinosylvin. Abbreviations: C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:coenzyme A ligase; PAL, phenylalanine ammonia-lyase; STS, stilbene synthase.

Pinosylvin is a 3,5-dihydroxystilbene. It is formed under developmental control, constitutively, in the heartwood or as phytoalexin in the sapwood and needles after induction by stresses in many tree-species, especially of the genus *Pinus* (Erdtman 1939, Rennerfelt 1945, Erdtman et al. 1951, Lindstedt 1951, Lindstedt and Misiorny 1951, Rosemann et al. 1991). Resveratrol is a 3,4',5-trihydroxystilbene accumulating in response to fungal infection and known to reduce the growth of fungi pathogenic to grapevine such as *Botrytis cinerea* (Hoos and Blaich 1990, Adrian et al. 1997, Paul et al. 1998), *Rhizopus stolonifer* (Sarig et al. 1997) or *Phomopsis viticola* (Hoos and Blaich 1990). The difference in antifungal activity between these two stilbenes still needs to be elucidated. The mechanism of toxity of stilbenes is not well understood either but they may act by inactivating enzymes containg –SH groups in their active sites (Hart and Hillis 1974).

The role of stilbenes in disease resistance has become more evident through gene transfers. Expression of STS enhanced the resistance of tomato, barley and grapevine to *Botrytis cinerea* (Hain et al. 1993, Leckband and Lörz 1998, Coutos-Thévenot et al. 2001), of tomato to *Phytophthora infestans* (Thomzik et al. 1997), of rice to *Pyricularia oryzae* (Stark-Lorenzen et al. 1997) and of alfalfa to *Phoma medicaginis* (Hipskind and Paiva 1998). All the experiments described above have employed grapevine or peanut stilbene synthase genes in gene transfers, and only one report has shown the expression of a pinosylvin synthase gene in a new plant species. However, none of the tested wheat lines expressing pinosylvin synthase gene from pine showed a reduction in disease incidence (Serazetdinova et al. 2005). Recently, stilbene synthase from grapevine has been transferred to several tree species, namely kiwi (Kobayashi et al. 2000), apple (Szankowski et al. 2003), papaya (Zhu et al. 2004) and white poplar (Giorcelli et al. 2004). In tree species, the effects of grapevine-STS gene transfer on resistance have not been as promising as with herbaceous species. Transgenic trees accumulated resveratrol glycosides but an increased resistance was only reported in papaya against diseases caused by *Phytophthora palmivora* (Zhu et al. 2004). Stilbenes are generally isolated from plants in a glycoside form (Hart 1981), piceid, which may not have the same antifungal activity as stilbene aglycones (Kobayashi et al. 2000, Giorcelli et al. 2004). Another possible explanation might be the variation in disease resistance against different pathogens.

1.4. RISKS RELATED TO TRANSGENIC TREES

Despite the fact that the advances provided by genetically engineered trees may be significant, there are many concerns about the impact of genetically modified (GM) trees on the environment. Genetic engineering may produce organisms that are in some way novel to an existing network of ecological relationships. Environmental risks associated with transgenic trees are specific to the gene inserted, the tree species, and the environment the tree is living in, so the risks related to a transgenic organism should be evaluated on a case-by-case basis (Wolfenbarger and Phifer 2000). Cultivations of trees are in many aspects different from cultivations of crop species since trees are long-lived (need for stable, long-term expression of transgenes), genetically close and growing in proximity to their wild relatives, outbreeding, and quite often wind-pollinated (spread of pollen and seeds) (Mathews and Campbell 2000, Burdon and Walter 2004).

A risk can be defined as a function of the probability of a negative effect occurring and its seriousness (Burdon and Walter 2004). The possibility that transgenic plants would hybridize with wild-type plants, is one of the most frequently mentioned risks among genetically modified plants (Mathews and Campbell 2000, Wolfenbarger and Phifer 2000, Conner et al. 2003). Genetic manipulation of wind pollinated and wind dispersed forest trees could easily disrupt natural community dynamics, thus an approach to link genes that inhibit flowering with the transgene of interest could prevent the escape of transgenes into natural ecosystems. Transgenes conferring resistance to pests, diseases, and herbicides could result in enhanced fitness, survival and spread of transgenic plants and hybrids making them highly invasive (Wolfenbarger and Phifer 2000, Ellstrand 2001). Some frequently mentioned risks of GM plants also include the potential evolution of new pests resistant to toxins produced in transgenic plants (Mathews and Campbell 2000, Ellstrand 2001), the instability of transgene expression especially in long-lived trees (van Frankenhuyzen and Beardmore 2004) and the risk of creating new potential allergens (Wal 2001). At least theoretically, transgenes from GM plants could be horizontally transferred to other (sexually incompatible) organisms such as terrestrial bacteria. Experimental approaches in both field and laboratory studies have not been able to confirm the occurrence of horizontal gene transfer (HGT) to naturally occurring bacteria (Nielsen et al. 1998), although HGT from plants to a soil bacterium has been reported in the presence of sequence homology between the donor and the recipient (Tepfer et al. 2003). The plasmid elements (promoters, selection markers etc.) having bacterial origin may facilitate HGT from transgenic plants to bacteria, however, it seems that the frequencies of successful HGT from plants may be exceptionally low and requires an extremely long time scale (Nielsen et al. 1998).

The complexity of ecological systems presents a considerable challenge for assessing the risks and benefits of genetically modified plants (Wolfenbarger and Phifer 2000). Constitutive expression of toxic compounds may affect not only the target pathogen, but also beneficial micro-organisms such as mycorrhizae, rhizobia and other micro-organisms involved in plant health, litter decomposition and nutrient cycling (Glandorf et al. 1997) or any species that feed on engineered plants (Conner et al. 2003). It is even more difficult to study the indirect effects of GM plants on non-target organisms e.g. via the accumulation of the toxic transgene products in food chains or population changes of species feeding on the target pests (Wolfenbarger and Phifer 2000, Conner et al. 2003). In

a few cases, changes in the populations of bacteria, fungi and soil invertebrates have been discovered but the causes of the differences have remained unproven (Conner et al. 2003). Pleiotropy, the condition in which a single gene affects multiple traits, can cause changes in plant characteristics that are difficult to predict. These pleiotropic effects also create challenges to the risk assessment of genetically modified organisms.

1.4.1. Interactions with mycorrhizal fungi and soil decomposers

Trees form symbiotic associations with a number of mycorrhizal fungi that facilitate water and nutrient supply and provide protection against pathogenic attacks. The majority of fungi colonising the fine roots of trees in boreal forests are ectomycorrhizas (ECM) which in addition to birches establish e.g. with *Pinaceae*, *Fagaceae*, and *Salicaceae*. During the formation of an ectomycorrhiza the fungus forms a structure called the mantle enclosing the rootlet and penetrates intercellulary between the epidermal and cortical cells to develop the so-called Hartig net (Harley and Smith 1983, Smith and Read 1997). Mycorrhizal fungi involved in plant-fungus symbioses contain chitin in their cell walls (Smith and Gianinazzi-Pearson 1988). Mycorrhiza formation triggers the induction of various defence-related proteins in the host plants including the increase in chitinase activities (Albrecht et al. 1994a, b). However this activation is only transient and occurs during early stages of mycorrhiza establishment (Volpin et al. 1994, Gianinazzi-Pearson et al. 1996). In ECM formation, wall-localised/apoplastic chitinases like sugar beet chitinase IV may be part of an early defence response and may function to degrade chitin fragments, released from walls of the symbiontic fungus, reducing the defence reactions of the plant and allowing symbiotic interactions of both organisms (Collinge et al. 1993, Salzer et al. 1997a and b).

The overexpression of chitinases in the roots of the genetically modified host plant could impede mycorrhizal development by switching the plant interaction from accommodating to defensive mode. Intercellular hyphae should readily come into contact with extracellular chitinase expressed constitutively in transgenic plants. The effects of the expression of additional chitinases on the establishment of mycorrhizal symbiosis have been studied in transgenic tobacco plants constitutively expressing different forms of chitinase. Transgenic plants were equally well colonized by the mycorrhizal fungus *Glomus mossaeae* as the control plants indicating that the transgenic expression of chitinase

did not interfere with the establishment of vesicular-arbuscular mycorrhizal symbiosis (Vierheilig et al. 1993, 1995). On the contrary, a delay in colonisation was observed in tobacco plants constitutively expressing PR-2 gene with β-1,3-glucanase activity showing that beneficial symbiotic fungi can be affected adversely in plants expressing PR's for enhanced pathogen resistance (Vierheilig et al. 1995). Since lignin has an important role in plant defence as a structural barrier, lignin modifications might also cause changes in the interactions between transgenic plants and the beneficial and pathogenic fungi.

Plants provide the resources for the functioning of decomposer subsystem therefore the quality and quantity of aboveground plant material may have important influence on components of the soil biota (Wardle et al. 2004). Effects of the products encoded by various transgenes on non-target organisms in the soil have been considered as potential risks of genetically engineered plants (Glandorf et al. 1997). Decomposition of transgenic plants has been studied under laboratory conditions and in the field, and the studies have shown varying results about the influence of genetic manipulation on litter degradation. In addition, many of the studies are likely to reflect pleiotropic effects due to genetic modifications, rather than the effects of transgenes per se (Vauramo et al. 2006).

1.4.2. Pleiotropic effects

During transformation, foreign DNA integrates randomly into the plant genome (Puchta and Hohn 1996). Expression of a transgene is influenced by the integration site and the copy number of the integrated transgene (Meyer 1995, Kumar and Fladung 2001). On the other hand, introduced genes can suppress or accelerate the expression of endogenous genes and/or transgenes already present in the genome and thus regulate many apparently independent properties in an organism (Taylor 1997, Käppeli and Auberson 1998). Pleiotropic changes in plant characteristics such as vegetative and flower development as a result of the transformation process have been reported in several studies (Elkind et al. 1990, Austin et al. 1995, Ahuja and Fladung 1996, Romero et al. 1997, Donegan et al. 1999, Gutiérrez-Campos et al. 2001, Lemmetyinen et al. 2004). The contribution of position effects to unexpected secondary effects in transgenic plants may be minimized by developing techniques for site-directed gene insertion (Käppeli and Auberson 1998).

Non-target effects of transgenic plants on soil organisms may also be due to pleiotropic effects of the transgene. Unintentional changes in plant characteristics such as shoot weight or nutrient levels in the plant tissue resulting from genetic manipulation may have impact on soil chemistry and microbial community (Donegan et al. 1997, Donegan et al. 1999). Changes in soil bacterial communities and enzyme activities associated with lignin peroxidase transgenic alfalfa plants showing lower shoot weight and higher N and P content (Donegan et al. 1999) and growth and species composition of soil microorganisms associated with transgenic cotton expressing *Btk* (*Bacillus thuringiensis* var. *kurstaki*) endotoxin (Donegan et al. 1995) have been explained to be partly due to pleiotropic effects of the transgene. The faster decomposition rate of chitinase transgenic silver birch leaves also seems to derive from pleiotropic effects of the transgene on the structural components of the leaves (Kotilainen et al. 2004).

1.5. FIELD TESTS WITH TRANSGENIC TREES

In order to study the stability of the introduced characteristics and to assess the environmental impacts of transgenic trees, field trials have been established in several European countries, including Finland, France, Germany, Italy, the Netherlands, Spain, Sweden and the UK. These involve transgenic varieties of olive, plum, apple and orange, but also forest trees like birch, spruce, pine, eucalyptus, and different *Populus* species. In the United States and China field trials with transgenic trees have started earlier than in Europe (Aronen 2002). The majority of the permit applications for field releases are related to herbicide tolerance (32%), marker genes (27%), insect resistance (12%), and lignin modifications (9%). Relatively little research has been conducted to date on ecological risks associated with the use of transgenic trees. Collecting the data needed to realistically assess risks and benefits of transgenic trees has been constrained by current regulations allowing only small-scale and short-term testing in the field as field releases are not permitted to continue beyond the minimum time required for sexual maturity and flowering (van Frankenhuyzen and Beardmore 2004). It is rather likely that a range of transgenic trees will be introduced into forestry use in some parts of the world. So far, virus-resistant papaya in Hawaii and insect-resistant poplar expressing *Bt* gene in China are the only authorized transgenic trees commercially released (Meilan et al. 2004, Strauss 2004).

2. AIMS OF THE STUDY

We produced transgenic birch and *Populus* lines in order to study the possibilities of engineering important forest tree species in Finland for disease resistance and wood properties. To assess the advantages and environmental risks associated with gene transfers, interbiontic processes between transgenic trees and pathogenic and symbiotic fungi and soil decomposers were studied *in vitro* and in field conditions.

The more specific aims of the present study were:

1) To open the stilbene pathway leading to the synthesis of pinosylvin in aspen and hybrid aspen and to evaluate the effect of transformation on decay resistance of transgenic trees.
2) To study the disease resistance of chitinase transgenic silver birches expressing different levels of the transgene against natural infection of foliar pathogens in a field trial.
3) To study the effect of constitutive chitinase overexpression on mycorrhiza forming ability of transgenic birch lines *in vitro*.
4) To produce transgenic birch lines with altered lignin properties by 4CL antisense gene transformation and to study the effects of genetic manipulation on plant characteristics and interactions between transgenic trees, mycorrhizal fungi and soil micro-organisms.

3. MATERIAL AND METHODS

This section provides only the summary of the used methods. More detailed description of the material and methods are found in the original papers I – IV, or in the original articles cited in them. The gene transformations and the assayed parameters have been summarized in Table 1.

Table 1. A summary of the contents of publications I – IV.

Gene transformation	Transformed trait	Methods and assayed parameters	Publication
STS	**Disease resistance**	PCR	**I**
		Southern blot analysis	**I**
		Northern blot analysis	**I**
		STS enzyme activity	**I**
		Decay resistance	**I**
Chitinase	**Disease resistance**	Northern blot analysis	**II, III**
		Disease scoring in the field: *Number and % of leaf area covered by leaf spots or rust postules; general disease score*	**II**
		In vitro mycorrhiza-experiment: *Number of root tips; % of mycorrhizal root tips; root, shoot and total FW; root/shoot ratio*	**III**
Antisense 4CL	**Lignin biosynthesis**	PCR	**IV**
		Southern blot analysis	**IV**
		Lignin and cellulose contents, composition of lignin	**IV**
		Stem height and root FW	**IV**
		In vitro mycorrhiza-experiment: *Number of root tips; % of mycorrhizal root tips; root and shoot FW; root/shoot ratio*	**IV**
		Leaf decomposition: *Mass loss; basal and substrate induced respiration; ergosterol content*	**IV**

3.1. PLANT MATERIAL

Seedlings of aspen (*Populus tremula*) and hybrid aspen (*Populus tremula* × *tremuloides*) clones 1 and 51 (controls and the transgenic lines) (I), 15 chitinase transgenic lines from an elite Finnish birch (*Betula pendula* Roth) clone JR1/4 and the non-transgenic control (II, III) and transgenic seedlings and the controls from early flowering birch clone BPM5 (IV) were propagated and rooted *in vitro*. Transgenic birch lines and their controls were multiplied on 3/4 MS medium (Murashige and Skoog 1962) with 1 mg/l BAP and rooted on a rooting media (1/2 MS with 0.5 mg/l IAA) (II, III, IV). Transgenic aspen and hybrid aspen lines and their controls were propagated on WPM medium (Lloyd and McCown 1980) with 0.2 mg/l BAP and rooted as seedlings of birch (I). Seedlings with induced roots were planted on soil and grown in the greenhouse (I, II, IV). In autumn 2000, after 18 months in the greenhouse, 15 replicates of 15 chitinase transgenic silver birch lines and the corresponding non-transgenic controls were planted in the field as a randomised block design (II).

3.2. VECTOR CONSTRUCTIONS

For stilbene synthase gene transfer, a transformation vector pSKS14 was constructed containing a *Pinus sylvestris* stilbene synthase gene (courtesy of Dr. Joachim Schröder, Freiburg, Germany) under the control of 4x35S-promoter (I). The binary plasmid pBKL4K4 containing a sugar beet chitinase IV gene with the enhanced (4x) CaMV 35S promoter was a gift of Dr. J. D. Mikkelsen (Danisco) (II, III). *Betula pendula* 4CL1 (*Bp*4CL1) cDNA was isolated from total RNA by RT-PCR using degenerate oligonucleotide primers based on conserved amino acid domains. A 836 bp cDNA fragment was cloned in antisense orientation downstream of a CaMV 35S-promoter in the pBI121 binary vector (Clontech, Franklin Lakes, NJ, USA) (IV). All the constructs carry the *nptII* (neomycin phosphotransferase) gene for kanamycin selection of transgenic plants.

3.3. GENE TRANSFER TO ASPEN AND BIRCH

Aspen and hybrid aspen clones were transformed using *Agrobacterium*-mediated gene transfer. Vector pSKS14 was conjugated into the disarmed *Agrobacterium tumefaciens* strain

C58C1 (pGV2260) by triparental mating using the *E. coli* RK2013 as helper. Pieces of *in vitro* -grown *Populus* leaves and stem were co-cultured with *Agrobacterium* on supplemented MS-medium to let the agro-infection to occur. After two days of co-culture, *Agrobacterium* was eliminated by rinsing the explants in washing solution for five days before transferring the explants to selection plates containing kanamycin, cefotaxime (Claforan) and vancomycin. Shoots emerging from the transformed explants were cut off and grown in modified WPM supplemented with kanamycin (I).

Transformation of birch was performed essentially as described in Keinonen-Mettälä et al. (1998) and Keinonen (1999) (see Pappinen et al. 2002). *Agrobacterium* strains LBA4404 (pAL4404) and C58C1 (pGV2260) were used to introduce the sugar beet chitinase IV and *Bp*4CL1 genes in binary plasmids pBKL4K4 and pBI121 into the birch clones JR1/4 and BPM2 or BPM5, respectively. Explants from sterile *in vitro* plants were pre-cultured for five days before the gene transfer. After preculture, the explants were mechanically wounded and co-cultured with *Agrobacterium* for 3-5 days in liquid MS or MSG (Brown and Lawrence 1968) medium. To wash away any residual *Agrobacterium*, the explants were rinsed for 5-7 days in MS-medium containing claforan and vancomycin. Transgenic tissue was selected on WPM medium supplemented with claforan, ticarcillin, and kanamycin. Adventitious shoots were further selected on WPM medium containing kanamycin (II, III, IV).

3.4. DNA EXTRACTION

Total DNA from transgenic and control plants was isolated according to Lodhi et al. (1994) with some modifications (see Pappinen et al. 2002). Plant material was frozen in liquid nitrogen and ground using mortar and pestle. The resulting powder was suspended in preheated extraction CTAB buffer with β-mercaptoethanol and PVP, and the mixture was incubated at 60ºC for 30 minutes. After cooling, the mixture was extracted with an equal volume of chloroform:isoamyl alcohol (24:1) and following centrifugation, a half-volume of CTAB buffer was added to the resulting supernatant. Incubation and extraction with chloroform:isoamyl alcohol were repeated, and the DNA was precipitated with absolute ethanol. The precipitated DNA was resuspended in CTAB buffer, the mixture was incubated and extracted with chloroform:isoamyl alcohol; these extraction steps were repeated as previously until a white interface was no longer visible.

Finally, the DNA was precipitated and washed with ethanol and resuspended in a TE solution (I, IV).

3.5. RNA EXTRACTION

Total RNA was extracted from leaves and *in vitro* roots ground in liquid nitrogen as described by Chang et al. (1993). Preheated extraction buffer was added to the powdered plant material, the tubes were incubated in a water bath (65ºC) and shaken vigorously for at least 15 minutes. The mixture was extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1), and ¼ volume of 8 M lithium chloride was added to the second supernatant. The RNA was precipitated overnight at 4ºC and harvested by centrifugation. The pellet was dissolved into SSTE solution, and the mixture was extracted once with chloroform:isoamyl alcohol. The RNA was reprecipitated by adding absolute ethanol to the supernatant and after centrifugation, the pellet was suspended in sterile water. Total RNA was quantified spectrophotometrically by measuring A_{260} (I, II, III).

3.6. POLYMERASE CHAIN REACTION

Transfer of the pine stilbene synthase gene into aspen and hybrid aspen was initially scored by PCR using two oligonucleotides
PINOFOR (5'- GGAAGTTGCAGAGGGCAGATG-3') and
PINOREV (5'-CCACCGATGGCTCCCTCGCTG-3') which amplify a 746 bp fragment of the transferred sequence. The PCR reaction has been described in paper I. A plasmid containing *Pinus*-STS was used as a positive control, non-transgenic aspen and hybrid aspen acted as negative controls for the PCR.

The screening of putative transformants for the integration of *Bp*4CL1 gene in birch was carried out by PCR using primers specific for the kanamycin resistance gene
*nptII*fw (5'-GCTTGGGTGGAGAGGCTATT-3') and
*nptII*rev (5'-GCGATACCGTAAAGCACGAG-3') which amplify a 701 bp fragment of the transferred sequence. Plasmid pRT104 carrying the *nptII*-gene and DNA from the non-transgenic birch clones BPM2 and BPM5 served as positive and negative controls, respectively (IV).

3.7. SOUTHERN BLOT ANALYSIS

For the Southern analysis, 8 μg of DNA from aspen and hybrid aspen transformants and their controls was digested overnight with the restriction enzyme *Nde*I (I); 15 μg of DNA isolated from the antisense birch lines and the untransformed wild type was digested with the restriction enzyme *Eco*RI (IV). After size-fractionation on a 0.8% agarose-gel, the DNA in the gel was depurinated, denaturated, neutralised, and blotted to a positively charged nylon membrane as described by the manufacturer Roche Diagnostics (Rotkreuz, Switzerland) (I, IV). In paper I, a pine stilbene synthase 1356 bp fragment labelled with the DIG-High Prime kit (Roche Diagnostics) was used as a probe. Prehybridisation, hybridisation, posthybridisation washes and detection were carried out as described (I). For Southern analysis of birch, a 701 bp *nptII* fragment was labelled using [^{32}P]dCTP with the *redi*prime^{TM}II random prime labelling system and purified by NICK^{TM} column containing Sephadex G-50 according to manufacturer's instructions (Amersham Pharmacia Biotech, UK). The membrane was prehybridised at 42ºC for 2 hours or more in ULTRAhyb^{TM} hybridisation buffer (Ambion, UK) and after adding the probe, hybridised overnight in the same buffer and temperature. Membranes were washed once with 6 x SSC for 5 min, twice with 2 x SSC, 0.1% SDS for 20 min, and twice with 0.2 x SSC, 0.1% SDS for 20 min at 62ºC. The signal was detected by exposing the membrane to Kodak BioMax MS film (IV).

3.8. NORTHERN BLOT ANALYSIS

To detect the expression of the introduced genes total RNA was separated on a denaturing 1% agarose gel in MOPS-buffer and capillary-blotted onto positively charged nylon membranes (Roche Diagnostics). The membranes were hybridised with digoxigenin-labelled chitinase (500 bp) (II, III) or stilbene synthase (1356 bp) (I) probes. Prehybridisations, hybridizations, and high stringency washes were performed as in Church and Gilbert (1984) with a few modifications and have been described in paper I. Detection of the chemiluminescent signal was carried out according to manufacturer's (Roche) instructions. The membranes carrying the hybridized probe and antidigoxigenin Fab fragments conjugated to alkaline phosphatase bound to the hybridized probe were reacted with the chemiluminescent substrate. To record the signal, the membranes were exposed to Fuji RX X-ray films (I, II, III).

3.9. STS ENZYME ASSAY

Stilbene synthase enzyme activities were assayed according to Fliegmann et al. (1992). Crude plant extracts were incubated with cinnamoyl-CoA and [2-^{14}C]-malonyl-CoA for 20 minutes at 37ºC. The products were separated by thin-layer chromatography and identified by co-migration with the products of pine STS expressed in *E. coli*. Detection was carried out with a Phosphoimager (I).

3.10. DECAY TEST WITH *PHELLINUS TREMULAE*

Sterilised wood samples from transgenic and control aspen and hybrid aspen trees were decayed for 7-8 weeks with the white-rot fungus *Phellinus tremulae* on malt agar plates. After incubation, the growth type of the fungus (bleaching or staining) and weight loss during the decay test was recorded (I).

3.11. FIELD TRIAL

The field trial in Viikki area in Helsinki of 8 untransformed silver birch clones and silver birch lines genetically modified for chitinase (15 lines) and other properties such as peroxidase and chalcone synthase, was carried out by the permission from the Board of Gene Technology (notification number 2/MB/00), and the regulations concerning the safe handling of GM-material were applied (Directive 2001/18 EC). The monitoring period of the field trial lasted three years, from 2001 to September 2003. The field trial was harvested in October, 2003 (II).

3.11.1. Disease scoring in the field trial

The symptoms of the natural infection of two fungal diseases, birch rust *Melampsoridium betulinum* and leaf spot disease *Pyrenopeziza betulicola*, were analysed from the leaves of chitinase transgenic lines and the wild-type control JR1/4 each year in September 2001, 2002, and 2003. Three parameters were estimated; the number of disease spots per leaf, the percentage of the leaf area covered by disease spots, and a general disease score (II).

3.12. *IN VITRO* MYCORRHIZA EXPERIMENT

In vitro inoculation of birch was performed according to Timonen et al. (1993). Chitinase transgenic and 4CL antisense birch lines and the corresponding controls were rooted *in vitro* and transferred to test tubes containing solidified Brown and Wilkins' media (Brown and Wilkins 1985) and Leca clay particles. Plants were inoculated with *Paxillus involutus* by placing two mycelium discs into test tubes in sterile conditions, part of the plants were left as controls without inoculation. The tubes were sealed with a cotton cap, the lower part of the tubes was covered, and the seedlings were grown in a growth chamber. The experiments were harvested after 4-6 weeks of inoculation when roots and shoots were separately weighed and the total number of mycorrhizal and non-mycorrhizal root tips was counted (III, IV).

3.13. WOOD PROPERTIES

Analysis of the lignin and carbohydrate contents of the antisense *Bp*4CL1 lines and isolation of milled wood lignin (MWL) was performed by KCL services (Espoo, Finland). Lignin content was determined according to the TAPPI-T 222 method. Monosaccharide composition was measured after acid hydrolysis by liquid chromatography. The released monosaccharides were separated on an anion exchange column and quantified by pulsed amperometric detection (HPAEC-PAD). No correction factors were used to compensate hydrolytic losses. Polysaccharide composition including the content of cellulose was computed from monosaccharide composition according to Janson (1974). The abundance of main structural units of lignin was determined from Q-HSQC spectra (Heikkinen et al. 2003) (IV).

3.14. LEAF DECOMPOSITION EXPERIMENT

The decomposition of leaf litter from 4CL antisense birch lines was studied in a field trial. Leaf samples were collected from the greenhouse and dried at room temperature. The dry leaves were weighed and placed into litterbags, which were buried in the soil. After 7 or 11 months, the litterbags were collected from the field. The decomposition of the litters was investigated by studying litter mass loss, fungal biomass (litter ergosterol content) and total microbial biomass (substrate induced respiration) and their activity (basal respiration). The determination of mass loss was carried out from dried and

incinerated leaf material. From thawed wet samples, a substrate induced respiration assay was performed using a Nordgren respirometer, and before adding the substrate, glucose, basal respiration activity was measured. For the quantification of ergosterol, frozen leaf material was lyophilisated and pulverised in liquid nitrogen. Samples and standards were treated according to Axelsson et al. (1995). An internal standard was added to each sample and the analysis was accomplished by gas-chromatography mass-spectrometry as described in Axelsson et al. (1995) (IV).

3.15. DATA ANALYSIS

Due to numerous tests used in the analysis of data, statistics are described in the original papers I-IV.

4. RESULTS AND DISCUSSION

4.1. THE IMPACT OF GENE TRANSFERS ON RESISTANCE TO FUNGI

4.1.1. Effects of stilbene synthase gene transfer in *Populus*

Transgenic lines H4, H5 (aspen) and HH1.13, HH1.2, HH51VIII (hybrid aspen) were identified by PCR, Southern and Northern blotting, and stilbene synthase enzyme assay. The decay resistance of the transgenic lines against an important white-rot fungus of aspen, *Phellinus tremulae,* was assayed in *in vitro* test of wood discs introduced into fungus culture plates. When the weight loss percentages of the wood samples from the transgenic trees were compared to their controls after 7-8 weeks of incubation, lines HH1.13 and HH51VIII had decayed significantly faster than their non-transgenic controls while the aspen line H4 showed significantly increased tolerance to *P. tremulae*. Lines HH1.2 and H5 did not differ from their corresponding controls. In conclusion, no clear indication of an increased resistance was observed in the transgenic lines. The faster wood decay in transgenic lines HH1.13 and HH51VIII may be related to the growth type of the fungus; the plates with wood samples from these lines were mainly colonised by the fast growing bleaching type of the fungus while in the corresponding control plates the fungus developed more often as a slowly growing staining type. In the plate tests as well, control plates were colonised by the slowly growing type whereas in the presence of low concentrations of stilbenes *P. tremulae* grew as a fast growing type. In the decay test of aspen, only the plates with a bleaching type of *P. tremulae* were selected for the experiment thereby differences in the growth type of the fungus can not account for the observed increased resistance of transgenic line H4 to decay. Also, it has to be noted that in our study *P. tremulae* was among the most resilient against the stilbenes and the results might have been different with a more susceptible fungus (I).

Despite of the ability of our transgenic lines to synthesise active STS enzyme, we were unable to detect any pinosylvin, resveratrol or their glycosides in transgenic aspen or hybrid aspen (I). Although the chemical analyses did not show the accumulation of stilbenes, stilbene contents below the detection limit of our analytical methods might have been present. There are several possible reasons for lack of pinosylvin accumulation in the transgenic plants. Firstly, availability of the cinnamoyl-CoA for the reaction

catalysed by pinosylvin specific STS may be a factor limiting the synthesis of pinosylvin in some plant species. 4CL has a low, or not detectable, affinity for cinnamic acid in grapevine (Lofty et al. 1989), *Arabidopsis* (Ehlting et al. 1999) and *Pinus taeda* (Voo et al. 1995, Zhang and Chiang 1997). Also native poplar 4CL isoforms exhibited little or no activity with cinnamic acid (Grand et al. 1983, Allina et al. 1998). However, instead of its original substrate cinnamoyl-CoA, pinosylvin synthase accepts the more abundant p-coumaroyl-CoA as a substrate and catalyses the synthesis of resveratrol (Gehlert et al. 1990, Fliegmann et al. 1992). Still, other stilbene derivatives yet uncharacterised might be produced in the transformants as was the case in wheat transformed with pinosylvin- or resveratrol-forming STS which showed the accumulation of three unknown compounds in transgenic plants. The presence of three novel compounds in wheat suggests that STSs may have been unable to use their specific substrates and instead have utilised alternative substrates present in excess in plants (Serazetdinova et al. 2005). Secondly, stilbene synthesis in the transgenic plants may also be restricted by metabolic channelling of intermediates into different branches of phenylpropanoid metabolism and the inability of the introduced STS enzyme to enter the metabolic channel of enzymes organised into different membrane-associated multienzyme complexes (Weisshaar and Jenkins 1998, Winkel-Shirley 1999).

We were the first ones to report the expression of a pinosylvin synthase gene in a heterologous plant species (I), since results from only one study with pinosylvin expressing transformants have been published. None of the analysed wheat lines expressing pinosylvin-forming STS showed increased fungal resistance (Serazetdinova et al. 2005). Future experiments with new plants species, sophisticated analytical methods, and different plant pathogens are needed to evaluate the effectiveness of pinosylvin-forming-STS in engineering disease resistance. Also, the factors impeding the synthesis of pinosylvin in new host species need to be clarified before future attempts to enhance disease resistance by transfer of a pine STS.

4.1.2. Sugar beet chitinase expression in birch

The symptoms caused by natural infections of two major foliar pathogens of *Betula pendula*, the leaf spot fungus *Pyrenopeziza betulicola* (Fuckel) and the leaf rust fungus *Melampsoridium betulinum* (Kleb.), were evaluated in a field trial of 15 chitinase transgenic

birch lines during a period of three years. Both of these pathogens cause the premature yellowing and falling of leaves, which may reduce photosynthesis and have adverse effects on growth (Kurkela 1994). The resistance of the transgenic lines against leaf spot disease varied depending on the year and the studied parameter (number of disease spots, percentage of leaf area, general disease value). However, the general trend was that the individual transgenic lines were more susceptible to leaf spot than the non-transgenic control clone. This trend was consistent across the whole 3-year period indicating that the pattern of resistance did not fluctuate under varying weather and natural disease conditions. In contrast to leaf spot disease, the level of sugar beet chitinase IV expression improved the tolerance of birch against birch rust: the lines showing high or intermediate accumulation of the chitinase IV transcript were more resistant to birch rust than those showing a low accumulation. Among the individual lines, those differing significantly from the non-transgenic control clone with respect to rust tolerance were always more resistant to birch rust than the control (II).

Variation in the degree of resistance to different fungal diseases can be explained by differences in the biochemical composition and structure of the fungal cell wall, tissue and cellular localisation of the recombinant chitinase, concordance in chitinase expression kinetics and the period of infection, and the type of interaction between the plant and the pathogen (Grison et al. 1996). Different reactions to different pathogens were also shown in an *in vitro* assay where sugar beet chitinase IV inhibited the growth of the root-rot fungus *Heterobasidion annosum*, but no inhibiting effect on the growth of the scleroderris canker fungus, *Gremmeniella abietina*, was detected (Susi et al. 1995). In the case of the fungi analysed in this study the differential degree of resistance of chitinase transgenic birch against leaf spot and birch rust may partly be due to the totally different nature of these two fungi. Rust fungi are biotrophs that derive their nutrition from living plant cells and prefer to grow in vital host plants. The leaf spot fungus, in contrast, is a necrotrophic fungus living and proliferating in dead plant tissue (Kurkela 1994).

According to our previous studies, in the greenhouse conditions, chitinase IV expression in birch improved the resistance of the transgenic lines against the leaf spot fungus *P. betulicola* (Pappinen et al. 2002). Several potential causes could explain the contradictory results in the resistance of transgenic birch to leaf spot in the greenhouse and in the field trial. In the greenhouse experiment, a high inoculum of one isolate of *P. betulicola* was

used to infect the seedlings, while various genotypes of the same pathogen may have infected the transgenic birch in the field. Genetically distinct individuals have been detected in different spots within one leaf, indicating that multiple infection by *P. betulicola* naturally occurs on birch leaves (Paavolainen et al. 2000). The reduced resistance to leaf spot in the field may also have been caused by a variety of other factors such as stress caused by herbivorous insects, disturbances in mycorrhizal associations or changes in resource allocation which may not occur in the greenhouse under optimal growing conditions.

Previous studies have shown that the overexpression of chitinase genes can be used to increase the resistance of woody plant species to fungal pathogens (Bolar et al. 2000, Pappinen et al. 2002, Noël et al. 2005). Our results confirm these findings but emphasise the importance of disease resistance testing in natural conditions where test organisms are exposed to a variety of pathogens and abiotic stress conditions.

4.2. PROPERTIES OF 4CL ANTISENSE BIRCH

4.2.1. Molecular and lignin analysis

Molecular characterisation of the 4CL transformed antisense birch lines by PCR showed that three out of nine kanamycin resistant lines were transgenic. All the transgenic BPM5 lines F3, G1, and K3 possessed a single copy of the nptII gene when investigated by Southern blot analysis (Figure 3).

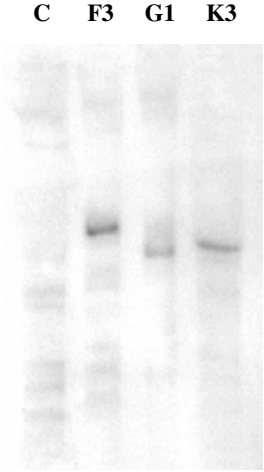

Figure 3. Southern blot analysis of *Eco*RI-digested non-transgenic control birch (C) and the 4CL antisense lines F3, G1, and K3. Digested samples were hybridised with ^{32}P-labeled 701 bp ntpII-fragment.

Transgenic lines contained fairly similar levels of total lignin and cellulose as the non-transgenic control clone BPM5, only the line K3 contained somewhat more lignin and less cellulose than the other transgenic lines and the non-transgenic control (IV). It is possible that the samples of line K3 may have included a greater proportion of older plant material compared to other samples which would explain the higher lignin content of line K3. Similarly, no differences in the abundance of main structural units of lignin between the antisense transgenic lines and the wild-type control were found (IV). The method using pooled stem samples in the chemical analyses of wood material was not optimal for detecting small differences in wood chemistry.

In the review by Anterola and Lewis (2002), results from three different studies of genetic downregulation of 4CL (Kajita et al. 1996, Lee et al. 1997, Hu et al. 1999) were recalculated to express lignin contents and 4CL activities as percentages of the controls. The comparison of lignin content with 4CL activity suggested that significant reductions in lignin contents occur only after > 60% reduction of 4CL activity. This implies that there is more 4CL activity available in the wild-type plants than necessary for the biosynthetic flux to the lignins and 4CL can thus not be considered to be a rate-limiting enzyme in lignin biosynthesis (Anterola and Lewis 2002). We presume that in the transgenic antisense birch lines analysed, 4CL-enzyme activity was not suppressed enough to reach the threshold to cause considerable changes in lignin content or composition. Also, although the cloned *Bp*4CL1 was considered as lignin specific by its sequence, no information exists about its substrate specificity or the total number of 4CL isoforms in birch.

4.2.2. Growth parameters

Unexpected growth reductions due to the expression of a transgene have been previously reported in a few studies (Elkind et al. 1990, Austin et al. 1995, Romero et al. 1997, Lemmetyinen et al. 2004). Our chitinase transgenic birch lines also showed adverse effects of genetic manipulation on growth parameters. Chitinase transgenic lines in *in vitro* mycorrhiza experiment usually had lower shoot weight than the controls (III). This data was confirmed in the field trial where the annual height and diameter increment of most of the transgenic lines was less than that of the non-transgenic control clone (Pasonen, personal communication). Similar data has been reported in apple where the expression

of a fungal chitinase gene increased resistance to apple scab but also reduced plant growth (Bolar et al. 2000). Chitinases may have a role in functions related to plant growth such as cell division, differentation, and development (Collinge et al. 1993, Sahai and Manocha 1993), which could partly explain the changes in the growing patterns of chitinase transgenic plants.

In the mycorrhiza experiment (IV), all the mycorrhizal 4CL antisense transgenic lines (except G1 in root/shoot –ratio and K3 in shoot fresh weight) showed significantly lower root and shoot fresh weight, root/shoot –ratio, and the total number of root tips compared to the mycorrhizal non-transgenic control clone. Similar variation between the transgenic lines and the control clone was detected among the non-mycorrhizal plants (Figure 4) suggesting that the changed growth pattern was not affected by mycorrhizal infection. In the greenhouse, the growth of transgenic line F3 was significantly lower compared to the non-transgenic control. All the 4CL antisense transgenic lines differed significantly from the control having less root biomass than the non-transgenic control (IV).

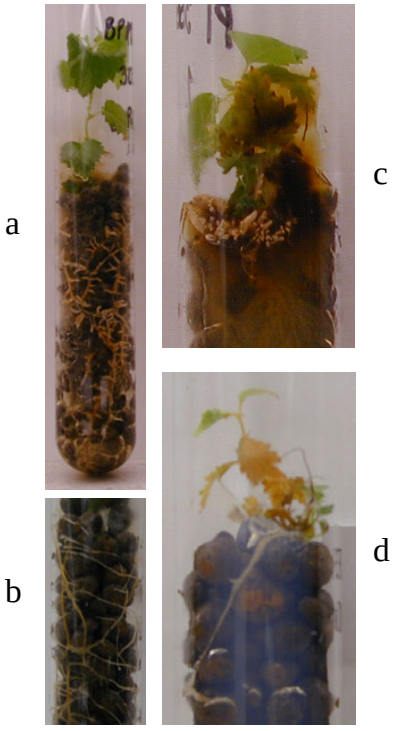

Figure 4. *In vitro* growth of the control line BPM5 (a, b) and the 4CL antisense transgenic line F3 (c, d) with (a, c) and without (b, d) *P. involutus* – inoculation.

In a study by O'Connell et al. (2002) transgenic tobacco plants suppressed in the activity of cinnamoyl-CoA reductase (CCR) showed changes in lignin structure and content but also a range of aberrant phenotypes. All lines with less than 50% of wild-type CCR activity displayed altered leaf morphology and many exhibited reduced growth. However, the correlation between the CCR activity of the transgenic lines, growth inhibition, and reduction in lignin content was not clear, as several transgenic lines with reduced height had normal levels of lignin while the most CCR-suppressed line had normal growth despite of a significant reduction in lignin content. Similarly, our 4CL antisense transgenic lines exhibited growth reductions without a change in lignin content (IV). When Kajita et al. (1996) introduced a sense 4CL into tobacco, dwarf stems were found at the flowering stage in three of the transgenic lines showing very low enzymatic 4CL activity. On the contrary, in transgenic aspens with antisense suppressed *Pt*4CL1 expression, leaf, root, and stem growth were substantially enhanced compared to control (Hu et al. 1999). Cell wall phenolic compounds that are derived from the same biosynthetic route as lignins may play important roles in the stimulation of growth and development of plant cells (Binns et al. 1987, Lynn et al. 1987), therefore alterations in lignin pathway may have pleiotropic effects as described above. The growth suppression of plants by chalcone has also been linked to the inhibition of 4CL activity, however further efforts are needed to clarify the potential of 4CL as a novel action site of the growth suppression (Yun et al. 2005).

Expression of a transgene can cause an energetic cost to an organism and divert energy and resources from cellular functions like growth, defence and reproduction. As a consequence of the metabolic load, the biochemistry and physiology of the host may be dramatically altered (Glick 1995, Purrington and Bergelson 1997). The fact that the prolonged callus culture phase and the synthetic growth regulators used in the production of transformants may yield individuals that are morphologically distinct from the original phenotype (Saieed et al. 1994) should also be acknowledged.

4.3. NON-TARGET EFFECTS OF TRANSGENIC BIRCH ON OTHER ORGANISMS

4.3.1. Mycorrhizal colonisation of transgenic birch

The ability of eight chitinase transgenic lines showing varying levels of sugar beet chitinase IV expression to form ectomycorrhizas was examined *in vitro*. Seedlings growing with the mycorrhizal fungus *P. involutus* had higher root / shoot –ratio than seedlings growing without the fungus (III). Increased allocation of carbon to the roots of mycorrhizal plants has been attributed to increased sink strength arising from colonisation by the fungal symbiont (Wright et al. 2000). The percentage of mycorrhizal root tips was lower in the transgenic lines 10 and 14, and in the group of lines showing high or intermediate sugar beet chitinase IV expression than in the non-transgenic control seedlings, although no statistically significant differences were detected (III). In contrast, when interactions between 4CL antisense birch lines and the mycorrhizal fungus *P. involutus* were studied, no changes were found in mycorrhizal formation due to the 4CL transformation. The data, however, showed that in all the lines including the non-transgenic control, mycorrhizal colonisation significantly increased the root/shoot ratio due to enhanced root growth and decreased shoot growth. Mycorrhizal plants had also greater number of root tips than the non-mycorrhizal plants although the difference was statistically significant only in line F3 (IV).

Fungal species vary in biochemical composition and the structure of the cell wall including the differences in chitin content, and the localisation and accessibility of chitin to chitinases, which may influence the ability of different fungi to resist plant chitinases (Grison et al. 1996). In *Brassica napus,* three fungal pathogens showed varying responses in the ability to infect transgenic *Brassica* plants constitutively overexpressing a hybrid tomato chitinase (Grison et al. 1996) indicating that differences may occur in the sensitivity of mycorrhizal fungi to transgenic chitinases. Since individual birch trees form ectomycorrhizal associations with a diverse community of fungal species in natural ecosystems (Fleming 1984, Newton 1991), the effects of the overexpression of chitinase in transgenic trees should be tested in a more natural-like environment with a wide diversity of mycorrhizal fungi. Considering the very good potential of *P. involutus* to form ectomycorrhizas and the deposition of sugar beet chitinase IV in the apoplast (Mikkelsen

et al. 1992), where it should easily make contact with fungal cell walls, a more specific fungus might have reacted differently.

The hyphae of ectomycorrhizal fungal species grow between the epidermal and cortical cells without penetrating the plant cell walls, where the lignin is deposited. Thus it is not unexpected that ectomycorrhizal fungi such as *P. involutus* do not seem to have the ability to decompose efficiently the lignocellulose matrix of cell walls (Colpaert and van Tichelen 1996). The majority of fungi colonising the fine roots of birch trees in boreal forests are ectomycorrhizas (Newton 1991) and changes in the composition of lignin or decrease in the lignin content might facilitate the growth of their hyphae in plant tissues. Due to the normal levels of lignin and despite the disturbed root formation in 4CL antisense lines, no clear differences between the transgenic lines and the control were observed in the colonisation of the roots by mycorrhizas (IV).

The slight decrease in mycorrhization among some chitinase transgenic lines combined with the lower shoot weight compared to the control plants (III) could lead to a decreased competitive ability of chitinase transgenic birch seedlings especially under harsh growing conditions. Field trials of transgenic trees provide a possibility to evaluate the ecological consequences of the effects of transgenes on mycorrhizas in a natural environment where the seedlings are exposed to a range of biotic and abiotic factors. As a part of our larger research project assessing the risks and possibilities of GM silver birches, root samples of chitinase transgenic and control lines have been collected from the field and the study on the rate of mycorrhiza formation and the diversity of fungal partners is in progress. Mycorrhizal colonisation of genetically modified hybrid aspen (*Populus tremula* × *P. tremuloides)* carrying an *A. rhizogenes* rolC gene has been investigated in a field trial. Despite the changed phytohormone balance in rolC-transgenic trees only minor and non-significant alteration of arbuscular mycorrhizas and ectomycorrhizas (EM) was observed in the field. In one transgenic line, minute physiological modifications not directly related to the function of the transferred gene resulted in a lowered frequency of one EM type. The role of hormones in mycorrhizal symbioses is not clear, however, it is important to rule out the effects of unexpected and unpredictable modifications on mycorrhization capability of transgenic plants (Kaldorf et al. 2002).

4.3.2. Leaf decomposition and soil organisms

In nature, trees interact with a broad spectrum of organisms, and genetically modified trees, if spread to nature, may have both direct and indirect effects on other species. Genetic engineering can cause foreseeable or unexpected consequences in plants that in turn can affect soil organisms and alter litter decomposition. The activity of fungi and bacteria, the main primary consumers of decomposable materials in soil, breaks down dead plant material and indirectly affects plant growth by determining the supply of available nutrients (Wardle 1999, Wardle et al. 2004). Decomposition of leaf litter is regulated by environmental conditions and the chemical nature of the litter such as the lignin or nitrogen content, the C:N ratio or the lignin:N ratio. Lignin controls the decomposition rate through its own resistance to enzymatic attack, and through physically interfering with decay of other chemical fractions of the leaf cell. As the decomposition proceeds, the proportion of lignin increases as microbes preferentially metabolize other chemical fractions readily available (Taylor et al. 1989).

In the case of plants with genetic modifications to lignin biosynthesis, risk assessment studies taking into account the effects on soil microbes and processes are fundamental because of the importance of lignin in regulating the rate of decomposition of plant material. Due to a reduction in the amount of lignin or a change in its composition or conformation, tobacco plants genetically modified for lignin biosynthesis decomposed more rapidly than material from the wild-type plants. The authors hypothesized that in the lignin-modified plants, the degree of protection offered by lignin to the associated polysaccharides might have been reduced (Hopkins et al. 2001). In contrast, in poplar trees genetic modifications to lignin biosynthesis (reduced CAD and COMT) had no significant effect on decomposition of woody trunk material (Tilston et al. 2004). Alfalfa plants genetically engineered for lignin peroxidase showed lower shoot weight and higher N and P content than the controls. In soil samples collected around the transgenic plants a lower activity of the soil enzymes dehydrogenase and alkaline phosphatase and an increase in the soil pH was detected; differences were also observed in the soil biota associated with the peroxidase engineered plants. The authors concluded that unintentional changes in plant characteristics appeared to have some impact on the soil ecosystem (Donegan et al. 1999). Any changes in plant productivity will in turn affect the carbon flux into the soil environment (Paterson et al. 1997), therefore the lower root or

shoot biomass of genetically modified plants could lead to decreased amount of resources to soil microbes. In our study, we wanted to find out if the decomposition of leaf litter from 4CL antisense transgenic lines differs from that of the litter from unmodified controls. When the percentage mass loss, the amount of microbial (SIR) and fungal biomass (ergosterol content) and the activity of microbial biomass (basal respiration) in the transgenic and wild-type birch litter were compared, significant differences were only found in the ergosterol content. The effects of the genetic transformation on the ergosterol content of the leaf litter were contradictory because the litter from one transgenic line (K3) had significantly higher ergosterol content and the litters from two other transgenic lines (G1 and F3) had significantly lower ergosterol contents than the litter from wild-type birch (IV). A shift in the composition of microbial communities in favour of fungi and against the Gram-positive bacteria was shown when residues from lignin-modified tobacco plants were decomposed in soil indicating that genetic modification of lignin biosynthesis can affect some structural attributes of the soil microbial community (Hénault et al. 2006).

5. CONCLUSIONS

In the future, the methods used in the biotechnology of crop species will eventually be employed in the breeding of forest species as the transformation and *in vitro* culture techniques develop. Our results show that disease resistance through genetic engineering of some native Finnish hardwood species can be achieved even though any practical applications are not expected in the near future. The high antifungal activity of pinosylvin makes the stilbene synthase gene from pine a good candidate for engineering disease resistance in heterologous plants if the factors limiting its synthesis are clarified. The data concerning the effectiveness of chitinase genes in conferring disease resistance in plants is extensive. Different reaction to two different leaf pathogens seen in our study emphasise the importance of testing the level of resistance of transgenic lines against various pathogens. The contradictory results in the resistance of chitinase transgenic birches to leaf spot in the greenhouse and in the field trial also clearly demonstrate the need and importance of field testing.

The potential ecological risks related to genetic engineering of forest trees include effects on non-target organisms such as mycorrhizas and soil biota and pleiotropic effects that are difficult to predict. According to the results of this study, in spite of a slight decrease in the percentage of mycorrhizal root tips among some chitinase transgenic lines, the expression of the antifungal transgenic chitinase in silver birch does not cause a threat to the symbiotic association between silver birch and *Paxillus involutus*. Results from the field test will further clarify the impact of the overexpression of the chitinase gene on the interaction between transgenic birch and different ectomycorrhizal fungal species colonising the roots of birch trees in natural conditions. Pleiotropic effects, e.g. changes in growth parameters, were observed in chitinase transgenic and 4CL antisense birch. In both cases, strong constitutive promoters of transgenes may cause metabolic load which could be avoided by applying inducible promoters. Future challenge is to restrict the expression of transgenes to a specific tissue or to a limited stage in the life cycle of a plant. The observed unexpected physiological changes of 4CL antisense lines did not disturb the interaction between *P. involutus* and the transgenic lines *in vitro* or the biodegradability of the plant material.

The results from these studies show the importance of field testing of the biotechnological applications for a variety of biotic and abiotic interactions. Diverse experimentation and long-term monitoring of transgenic trees is important to reveal the potential ecological effects even for transgenic forms that seem to be safe judging only from the primary effects of the transgene.

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